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# Optimization of conditions for vitrification of aqueous solutions of relevance to cryobiology

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# Optimization of Conditions for Vitrification of Aqueous Solutions of Relevance to Cryobiology

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By Michael J. Baker

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**Master's Thesis**

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# Abstract

Cryopreservation is a technology with wide ranging applications including helping to prevent species extinction (Hopkins and Herr, 2010). However, the current applicability of cryopreservation protocols is limited. This limitation warranted experimentation that could contribute to the optimization of current protocols for cryopreservation. The experiments presented herein were designed to contribute to the plunge-cooling method of cryopreservation. The importance for further development of current plunge-cooling methods is for the cryopreservation of cell-types sensitive to the other method of cryopreservation, slow-equilibrium freezing. The purpose for plunge-cooling is to cool a sample from ambient temperature to liquid nitrogen temperature at a fast rate. If cooling is sufficiently fast, ice-formation is inhibited. This creates a glass-like vitrified state.

To optimize the probability of vitrification, current approaches were modified. Different cooling methods were investigated. Containers called cryostraws were crafted from six different materials, materials with different thermal conductivity properties; all straws were of similar size. The minimum concentrations of several ice-inhibitors (cryoprotectants) needed to achieve vitrification were studied.

Novel cooling methods, which involved the use of spinning coolant and/or semi-solid nitrogen, called slush nitrogen (SN), as the cooling medium were invented and then tested. The novel methods enabled rates of cooling that were far faster than previously possible.

A chronic problem that plagued the progress with plunge cooling was the difficulty in making SN repeatable. The cause of this problem was researched and identified as contamination of the LN by condensed oxygen.

The quantified cooling rates of similar sized containers constructed from different materials yielded unexpected results. We determined that a material's thermal conductivity was a poor predictor of cooling rates. In fact, implementing a material that was porous to LN, such as polyvinyl chloride, yielded the fastest rate of cooling, indicating that a material's porosity is a much better predictor of cooling rate.

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# Introduction

The desired objective of cryobiology is to develop a means to indefinitely store biological cells, tissues and organisms in a way that maximizes their post-thaw viability potential. To succeed, intracellular ice formation must be prevented throughout the entire procedure — when the temperature is reduced to the storage temperature, during the storage period and during the thawing procedure. Although there are two generalized methods for cryopreservation, there are no universally applicable methods for successful cryopreservation. In general, the two current methods for cryopreservation fall into the category of either slow-equilibrium freezing or plunging-cooling. The latter method is the focus in this study; it is a particularly important approach when cell types are sensitive to cold and the slow-equilibrium freezing method is not effective. Such cell types can sometimes be successfully cryopreserved if frozen almost instantaneously and thawed likewise. However, current plunge-cooling methods are limited. It would be beneficial to broaden current plunge-cooling applications for those cell types sensitive to slow-equilibrium freezing.

We designed four experiments to optimize the conditions for successful vitrification of aqueous solutions when plunge-cooling. Currently, reports on the minimum concentration of cryoprotectants (CPs) needed for successful plunge-cooling vitrification of small volume samples are lacking in the literature (Berejnov *et al.* 2006). Therefore, the first experiment was designed to determine the minimum CP concentration required to vitrify a small volume in a container type consistent with what will work for freezing semen and small embryos. We studied cell-permeating CPs commonly used for cryopreservation: glycerol, ethylene glycol, dimethyl sulfoxide, and 1, 2-propandiol. Additionally, we studied isopropyl alcohol because of its similar molecular structure to the other four permeating CPs studied. The second experiment addressed the difficulties when producing SN after repeated cycles of freezing and thawing

liquid nitrogen (LN). The third experiment compared containers used for plunge-cooling. The containers were of the same size and shape, but were constructed from different materials. The fourth experiment quantified the cooling rate of a small volume sample plunge-cooled into different cryogenic mediums. Two novel methods for accelerating the rate of cooling of small volume samples were developed, which were then compared to current methods of cooling. Results from these experiments might contribute to an increase of cellular viability, post-thaw.

# Experiment 1

## Experimental determination of minimum concentrations necessary for solution vitrification when using cryoprotectants both singularly and in pairs

Cryopreservation of cells is performed by two general methods. One method is slow-equilibrium freezing. However, this subjects cells to lethal ice pressures generated externally (Fujikawa and Miura 1986; Ashwood-Smith, *et al.* 1988). Also, some types of cells are sensitive to cooling to 0 ˚C and below. An example is insect embryos that fail to survive because of this issue (Collins and Mazur, 2006; Mazur *et al.*, 1992). The second method, the focus of this study, is plunge-cooling. Plunge-cooling is implemented to avoid the disadvantages of slow-equilibrium freezing (Mazurs *et al.*, 1992; Steponkus *et al.*, 1990; Trad *et al.* 1999). It is performed by rapid plunging of cells into liquid nitrogen (LN) or slush nitrogen (SN). If the rate of cooling is adequately rapid for the transition to -136 ˚C, ice formation is inhibited. This method creates an amorphous state, free of intra- and extra-cellular ice crystals, and is commonly referred to as vitrification. The main disadvantage for plunge-cooling is the requirement of high concentrations of ice inhibitors called cryoprotectants (CPs) (Rall and fahy, 1985). Cells typically have limited tolerance to CP concentrations, with too high of concentrations being lethal (Lawson *et al.*, 2011 and Williams and Shaw, 1980). Lower, but sill adequate, concentrations of CPs for achieving vitrification are possible by increasing the cooling rates (Yavin and Arav, 2007; Risco *et al.*, 2007). Beyond this basic understanding, little is known about the relationship between CP concentrations when cooled at different rates.

An accepted theory is that CPs disrupt the hydrogen bonding networks of water molecules that form during crystallization (Kyrychenko and Dyubko, 2008). In this study, we tested biological-cell permeable CPs , singularly and in pairs, because there are instances when two or

more permeating CPs are implemented (Rall and Fahy 1985; Seki and Mazur 2009) to reduce individual CP concentration (Vajta *et al.*, 1998; Chian *et al.*, 2004). In 2007, Zhao *et al.* suggested that combining the permeating CPs ethylene glycol and dimethyl sulfoxide reduced toxicity in mouse embryo cryopreservation. No statistical difference occurred, however when combining ethylene glycol and dimethyl sulphoxide for ovine embryo vitrification (Dattena *et al.*, 2004). It is currently unknown if solutions containing two CPs provide synergistic effects in regards to the minimum concentration needed to vitrify the solution. The primary purpose of this study was to determine the minimum concentration of CPs needed to vitrify an aqueous solution in a container of a size useful for freezing such biological cells and organisms such as honey bee semen (Hopkins and Herr, 2010), *Drosophila melanogaster* embryos (our lab's interest), mammalian embryos (Chen *et al.*, 2001; Chian *et al.*, 2004) and other types of embryos and cells. Additionally, we investigated how the minimum required concentrations for vitrification were affected by the speed of cooling. Also, our study investigated if CPs act synergistically or are additive to affect the total minimum CP concentration for vitrification.

## Materials and Methods

### Methods for Cooling: Vapor-cooled or Plunge-cooled preparations

Two plunge-cooling methods were implemented and involved direct plunging of a quartz capillary tube , 7.5 cm in length (1.5 mm I.D. and 2 mm O.D.), into LN or into SN rotating on a turntable at 45 rpm. The latter cooling method is called turntable slush (TTS) (Baker and Herr, 2011). A third cooling method, vapor cooling, involved the placement of the ends of the quartz capillary onto a self-fabricated Styrofoam™ boat, which floated on the surface of LN. The Styrofoam™ boat was constructed from two separate pieces of Styrofoam™ (3.0 cm long x 0.5 cm wide x 1.9 cm high) connected with two pieces of 7.5 cm nickel chrome wire (28 awg) that

created a gap length of 6.5 cm. The gap length allowed the boiling LN vapor to be unimpeded towards rising and contacting the quartz capillary. The height of quartz above the LN when place atop the Styrofoam™ boat was ~ 1 cm.

Liquid nitrogen and TTS plunge-cooled quartz were plunged while holding the quartz perpendicular to the surface of LN or SN. Slush nitrogen was produced by placing 1.6 L of LN into a Dewar (11.5 cm inside diameter, 19 cm inside height, and ~ 1 to 2 cm wall thickness) that was placed into a self-fabricated vacuum chamber. A Hitachi cutevac™ pump (160VP N9028) provided a constant 23 in Hg vacuum. The process took between 10 to 30 minutes. Rotating SN on a turntable (General Electric 11-4022B) at 45rpm created the TTS effect. Quantification of cooling rates and minimum CPA concentration for vitrification for TTS was performed 2 to 5 minutes post removal from vacuum. This provided reproducible milkshake-like consistency of SN.

## Measurement of cooling rate

A t-type thermocouple (Omega Engineering, Inc. # TT-T-40-SLE)) with a wire diameter of 75 µm was inserted into a quartz capillary tube, which contained 25 µm of pure water (17.9 megohm) or a CP solution that consisted of 52.5 % v/v glycerol in DPBS. The junction of the thermocouple was placed mid-distant to the length of the liquid column. Temperature acquisition was recorded using National Instruments Labview™ software that controlled the National Instruments signal conditioning connector block (SC-2345). All temperature points were recorded at 2000 samples/s. Initially, data were acquired and manipulated with Labview™, and later analyzed using Microsoft's Excel™. The general protocol consisted of starting data recording at room temperature (21-23 $^{\circ}$C) and either plunge cooling into LN or TTS or vapor-cooling the sample. Each sample remained on the Styrofoam™ boat or below the

surface of LN and SN until the thermocouple reached the lowest temperature for each cooling method and then data recording was turned off. Samples were then removed from each cooling medium for warming. Manual expulsion of water or CP solution was next performed to allow for the loading of fresh water or CP solution into the quartz capillary tube. This process was repeated 10 times for the vapor-cooled method and 20 times for the plunge-cooled methods using LN or TTS. The temperature range for the cooling rate analysis was from 10 $^{\circ}$C to -150 $^{\circ}$C for each cooling method tested here. Latent heat of fusion of water was problematic when the cooling rates for the plunge-cooled methods were quantified. Thus, a 52.5 % v/v glycerol in DPBS solution was used for quantifying the cooling rates with the vapor-cool method; this concentration was vitrifiable.

Quantified cooling rates for the plunge-cooled methods were taken from a previous study; these were averaged and normalized to the slowest cooling rate in that experiment (LN) for pair-wise comparisons. Statistical analysis of the normalized cooling rates was performed by test statistics ($P < 0.05$) using small sample inferences concerning the differences between two means. The quantified cooling rates were from the temperature range of 10 $^{\circ}$C to -150 $^{\circ}$C (Baker and Herr, 2011).

**Cryoprotectant Solution formulation**

Five CPs were chosen: ethylene glycol (Fluka Chemika cat# 03750), dimethyl sulfoxide (DMSO) (ICN Biomedicals cat# 191418), glycerol (BDH cat# 101186M), 1, 2-propanediol (Sigma Aldrich cat# 398039-500 ml), and isopropyl alcohol (Sigma Aldrich cat#19030-500 ml). All CPs were formulated as 200 µl solutions in a glass test tube (10 mm by 75 mm), degassed for 60 s at 25 in Hg vacuum with a vacuum pump (GE motors 5KH33DN16GX) before loading into a quartz capillary tube and Crytosealed® for cooling. The single CP-containing solutions were tested in

concentrations ranging from 100 % pure CP, diluted in 5 % v/v increments with Dulbecco's Phosphate Buffer saline (DPBS) solution (Gibco™ cat#21300-017) until the CP concentration solutions no longer vitrified. The results obtained for the minimum CP concentration for vitrification when vapor-cooled or plunge-cooled into LN were used to formulate the binary CP concentrations in DPBS. The two CPs that would constitute a binary CP solution were formulated in direct proportion from their respective minimum single CP concentration required for vitrification when vapor-cooled or plunge-cooled into LN. This allowed the detection of positive or negative synergy or if binary concentrations simply acted additively in preventing ice formation.

In regards to this paper, synergy could mean that pairing 2 different CPs in a solution reduces the combined total required minimum CP concentration (positive synergy) for vitrification or if it increased the combined total required minimum CP concentration (negative synergy) for vitrification. An example of positive synergy would be if one CP by itself required 2 M to vitrify and the other CP by itself required 4 M to vitrify, when combined the minimum concentration needed to vitrify was less than 1 M of the one CP and less than 2 M for the other CP. An example of negative synergy would be the minimum concentration for vitrification requiring more than 1 M of the one CP and more than 2 M of the other CP. If the concentration when combined was 1 M of the one CP plus 2 M for the other CP to vitrify, it was considered to be an additive effect.

The formulation for binary CP in DPBS was as followed. Each of the CP concentrations that were the minimum required for vitrification in either vapor-cooled or LN plunge-cooled was considered a 1 X concentration. Then, 1.5 X concentrations were prepared for each of the CPs. Next, two different CPs both at 1.5 X concentrations were combined in equal volume. For

example, ethylene glycol and DMSO, both at 1.5 X concentration were combined in equal volume. This yielded one binary CP solution, which was 0.75 X, the concentration of ethylene glycol and 0.75 X concentration of DMSO that would be needed if used singularly. Hypothetically, if this binary solution, when further diluted with DPBS from a 0.75 X concentration to a concentration of 0.525 X for each CP, failed to achieve clear transparency, it would be an example of negative synergy. Additionally, if this binary CP solution could be diluted to a 0.475 X concentration or lower for each CP component and still achieve clear transparency when cooled; it would be an example of positive synergy. An additive effect for binary CP solutions would be if the required concentration for clear transparency was at a 0.50 X concentration for each CP. In these experiments, each CP component when in a binary CP solution was diluted with DPBS to create a range of concentrations from 0.75 X of each or total concentration of 1.5 X. The dilution reductions were in increments of 0.025 X (3.33 % v/v) for each CP in a binary CP solution until the solution failed to vitrify.

**Vitrification was determined by visual inspection after cooling to LN temperature**

Visual inspection of each CP solution was performed for the plunge-cooled methods at the surface of LN or SN. Visual inspections for the vapor-cooled CP solution were performed while the sample remained suspended on the Styrofoam™ boat. To facilitate visual inspections; a black background was positioned behind each solution (Berejnov, *et al.*, 2006; He, X., *et al.,* 2006) after the cooling process for each cooling method. An Energizer™ flashlight, which contained 2 “D” sized batteries, which powered 1 “incandescent” light bulb, provided further contrast of each inspected CP solution. Solutions of CPs were scored as apparent vitrification only if the solution was 100% clear transparent. The presence of blue tinting or small white

solids or a combination of both in the CP solution during inspection was scored as a failure to vitrify.

## Results

**Table 1-1 provides data showing the minimum required concentration (% v/v and M) for vitrification of a single CP in DPBS when cooled by vapor-cool (VC), liquid nitrogen (LN) or turntable slush (TTS).**

| Minimum CP Concentration (% v/v and M) Required for Vitrification of Single CP in DPBS | | | | | |
|---|---|---|---|---|---|
| cooling method -- cooling rate ˚C/min | DMSO | Glycerol | Ethylene glycol | 1, 2-propanediol | Isopropyl Alcohol |
| VC -- 125˚C/min | 45 %; 6.34 M | 45 %; 6.16 M | 40 %; 7.17 M | 40 %; 5.45 M | 35 %; 4.58 M |
| LN -- 1,593 ˚C /min | 40 %; 5.63 M | 35 %; 4.79 M | 40 %; 7.17 M | 30 %; 4.09 M | 30 %; 3.92 M |
| TTS -- 11,069 ˚C /min | 35 %; 4.93 M | 30 %; 4.11 M | 25 %; 4.48 M | 20 %; 2.72 M | 15 %; 1.96 M |

In Table 1-1, when the rate of cooling was accelerated, the minimum required CP concentration for vitrification was reduced for all CPs, except for ethylene glycol. Ethylene glycol required the same concentration when cooled with LN and VC. The greatest proportion of CP decrease was isopropyl alcohol; TTS could vitrify isopropyl alcohol with 57.21 % lower concentration than required to achieve vitrification with VC. Turntable slush reduced the required minimum concentration for 1, 2-propanediol, ethylene glycol, glycerol, and DMSO by

50.09 %, 35.52 %, 33.28 %, and 22.24 %, respectively.

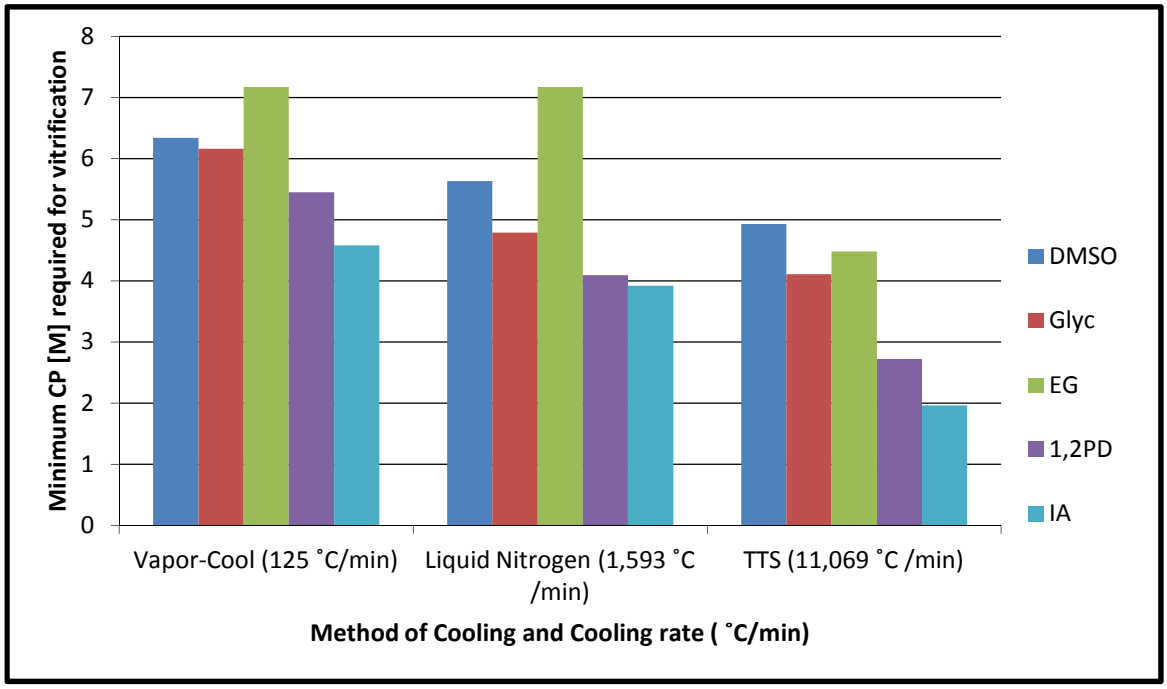


**Figure 1-1 presents the data for required minimum single CP concentration (M) for vitrification compared to three different cooling methods. Abbreviations: dimethyl sulfoxide is DMSO; glycerol is Glyc; ethylene glycol is EG; 1, 2-propanediol is 1,2PD; isopropyl alcohol is IA. The cooling rate presented here is the temperature range from 10 ˚C to -150 ˚C.**

The fastest method of cooling was TTS (Table 1-1 and Figure 1-1). Turntable slush cooled a small volume sample at 11,069 ˚C /min, which was almost 7 times faster than LN (1,593 ˚C /min) and 88 times faster than vapor cooling (125 ˚C /min). The TTS method for cooling also required the least concentrated CP solutions to achieve vitrification compared to the concentrations required for the other methods for cooling presented here. Vapor-cooling, the slowest method of cooling required the highest concentrations of all CP solutions to achieve vitrification.

Special consideration was given to isopropyl alcohol or DMSO as single CP solutions when determining the minimum concentration required for vitrification. Visual observations for isopropyl alcohol or DMSO were not consistent with the visual observations for the other single CP solutions tested. Visual observations for vapor-cooled or plunge-cooled with LN of isopropyl alcohol at concentrations ≥ 95 % v/v or ≥ 90 % v/v, respectively, resulted in completely clear vitrified transparency, which was similar to the other single CP solutions tested. However, 5 % v/v incremental dilutions with DPBS from concentrations of 90 % v/v to 40 % v/v for isopropyl alcohol, when vapor-cooled, resulted in cloudy/foggy white appearances. When the concentration of isopropyl alcohol was reduced to 35 %, 30 %, or 25 % v/v the solution appeared cloudy/foggy, cloudy/blue, or scattered white solids when vapor-cooled. We determined by visual observation that the minimum concentration for isopropyl alcohol in DPBS to achieve vapor-cool vitrification was at 35 % v/v, which was 4.58 M. Plunge-cooling isopropyl alcohol into LN reduced the concentrations when these types of appearances occurred.

For the LN cooling method, isopropyl alcohol in DPBS ranging in concentration from 85 % v/v to 40 % v/v appeared cloudy/foggy. However, when solutions of isopropyl alcohol of 35 %, 30 % or 25 % v/v were plunged into LN, a slight blue tint, or a clearness with marginal blue hue or slight clearness with prominent blue tint occurred, respectively. The blue tint/hue was translucent; much greater translucency than the cloudy/foggy appearance of the more concentrated isopropyl alcohol solutions, but again these solutions failed to achieve clear transparency. Because these solutions of isopropyl alcohol lacked clear transparency at concentrations relevant for cryopreservation, the minimum concentration for vitrification when plunge-cooled into LN is likely 30 % v/v, which is 3.92 M. Isopropyl alcohol required the lowest concentration, 1.96 M, among all CP solutions to vitrify when plunge-cooled with TTS.

The vapor-cooling or LN plunge-cooling of DMSO in DPBS at concentrations ≥ 85 % v/v or ≥ 90 % v/v, respectively, appeared 100 % cloudy; each solution was minimally translucent, not clear. The LN plunge-cooled DMSO solution at 85 % v/v was visually ~ 75 % cloudy and remainder with a distinguishable area of clear transparency. Further reduction of DMSO in DPBS when vapor-cooled or plunge-cooled into LN, vitrified 100 % clear transparency.

The CPs that achieved clear transparency as a single CP solution at all concentrations tested here were: ethylene glycol, glycerol, and 1, 2-propanediol. All binary CPA solutions appeared clear transparent.

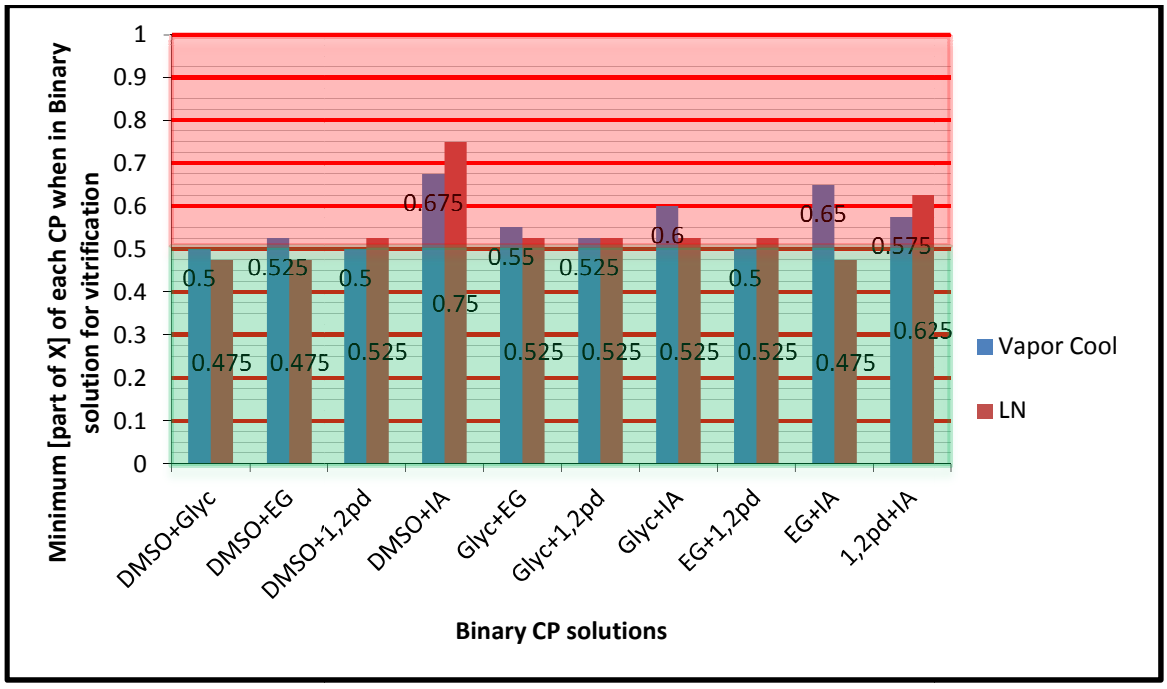


**Figure 1-2 presents the data for the minimum concentrations (proportion of X; 1 X is the expected concentration if the CPs were additive) required for successful vitrification of binary CP solutions when vapor-cooled or LN plunge-cooled. The CPs in each binary solution that have a minimum concentration above or below 0.5 X represents negative or positive synergy, respectively. If CPs were additive and not synergistic the bar would be found at 0.5 X. The red**

**area represents binary CPs that required higher CP concentrations than it would have been required if a single CP solution and the green area represents binary CPs that require lower CP concentrations than would have been required if a single CP was used.**

Three different combinations of binary CPs resulted in an additive effect when vapor-cooled. These combinations were DMSO + Glycerol, DMSO + 1, 2-propanediol, and ethylene glycol + 1, 2-propanediol. The highest degree of negative synergy with the vapor-cooled binary CPs tested all involved the use of isopropyl alcohol. Isopropyl alcohol paired with the CPs: 1, 2-propanediol, glycerol, ethylene glycol and DMSO, required a minimum of 0.575 X, 0.60 X, 0.65 X and 0.675 X concentration, respectively, to achieve vapor-cool vitrification. The increased cooling rate when plunge-cooling with LN only exacerbated the negative synergy with isopropyl alcohol when paired with either DMSO or 1, 2-propanediol. Isopropyl alcohol plus ethylene glycol yielded a positive synergistic effect, requiring 0.475 X concentration to achieve vitrification when plunged into LN. Two other LN plunge-cooled binary CP solutions resulted in a positive synergy; DMSO plus glycerol and DMSO plus ethylene glycol.

## Discussion

Five different CPs were prepared as single CP solutions and plunge-cooled into LN (see Table 1-1 and Figure 1-1). This was performed to determine the minimum required single CP concentration that would vitrify at a cooling rate of 1,596 ˚C/min. The CP solution that required the lowest minimum concentration for vitrification was isopropyl alcohol; it required 3.92 M. The CP solution that required the second lowest minimum concentration was 1, 2-propanediol; it required 4.09 M. The Glycerol, DMSO, and ethylene glycol solutions each required a minimum concentration of 4.79 M, 5.63 M, and 7.17 M, respectively, to vitrify (see Table 1-1 and Figure 1-1). The CP that required the least percentage to achieve vitrification when cooling in LN was

isopropyl alcohol; it required 30 %. The required minimum percentage of 1, 2-propanediol for vitrification using LN cooling was 30. The glycerol, DMSO, and ethylene glycol solutions required a minimum concentration of 35 %, 40 %, and 40 %, respectively (see Table 1). The smallest CP molecule tested here, isopropyl alcohol (M.W. 60.1 g/mol), vitrified in solution with the lowest required concentration (see Table 1-1 and Figure 1-1). However, the second smallest molecule, ethylene glycol (M.W. 62 g/mol), required the highest concentration in solution to vitrify. Interestingly, the largest molecule, glycerol (M.W. 92.09 g/mol), although it required a minimum concentration that was higher than both isopropyl alcohol and 1, 2-propanediol; glycerol required a minimum concentration that was lower than DMSO (M.W. 78.13 g/mol) and ethylene glycol to vitrify. There has been some suggestion that one of the mechanisms by which CPs prevent ice is by simply replacing part of the volume of water. These data are not consistent with that idea. The 1, 2-propanediol, which is a molecule that differs from isopropyl alcohol by having an additional alcohol group, required a higher CP concentration for vitrification than isopropyl alcohol (see Table 1-1 and Figure 1-1). Glycerol, which is a molecule that differs from 1, 2-propanediol by having an additional alcohol group, required a higher CP concentration for vitrification than 1, 2-propanediol. In this study, CPs with more alcohol groups had higher concentration requirements for the minimum CP concentration needed to vitrify when plunged into LN (see Table 1-1 and Figure 1-1).

The pairing of CPs for the binary CP solutions when cooled with LN yielded both positive and negative synergy in regards to the concentrations required to achieve vitrification. Interestingly, the binary CP solutions that yielded positive synergy included CPs that required higher concentrations when used singularly. The positive synergistic solutions were: DMSO paired with glycerol, DMSO paired with ethylene glycol, and isopropyl alcohol paired with ethylene glycol. These three binary CP solutions required a minimum concentration of 0.475 X

to achieve vitrification (see Figure 1-2). Seven binary CP solutions yielded negative synergy. Five of these seven were: DMSO plus 1, 2-propanediol; glycerol plus ethylene glycol; glycerol plus 1, 2-propanediol; glycerol plus isopropyl alcohol; and ethylene glycol plus 1, 2-propanediol. These five different pairs of CPs required a minimum concentration of 0.525 X to achieve vitrification. Two of the seven pairs of CPs required a minimum concentration that was ≥ 0.625 X to achieve vitrification. Pairs of CPs molecules that exhibited the greatest negative synergy surprisingly involved the CP: isopropyl alcohol. The pairing of isopropyl alcohol plus 1, 2-propanedial required a minimum concentration of 0.625 X to achieve vitrification when plunge-cooled into LN. The pairing of isopropyl alcohol plus DMSO required the *highest* minimum concentration of 0.75 X to achieve vitrification (see Figure 1-2). Interestingly, implementing the slower-cooling rate of vapor freezing for half of the binary CP solutions tested here yielded a lower required minimum concentration for vitrification (see Figure 1-2). Maybe CPs require time to allow for optimal positioning to prevent ice-formation.

The cooling rate affected the required concentration for vitrification of binary CP solutions. The pairing of isopropyl alcohol plus DMSO, which had the highest degree of negative synergy among all binary CP solutions when cooled with LN and vapor-cooled, required a lower minimum concentration of 0.675 X to vitrify when vapor-cooled (see Table 1-1). Three additional pairs of CPs, when vapor-cooled required lower concentrations to achieve vitrification than when cooled with LN (see Figure 1-2). There were five pairs of CPs when cooled with LN, instead when vapor-cooled, that required a lower concentration to achieve vitrification. The comparison of cooling methods had no effect on the required concentration for vitrification when pairing glycerol and 1, 2-propanediol (see Table 1-1).

Ethylene glycol, which required the highest single CP concentration to vitrify (see Table 1-1 or Figure 1-1), when paired with either DMSO or when paired with isopropyl alcohol and cooled with LN seemed to enhance vitrification; these pairings yielded positive synergy. However, when these pairs were cooled more slowly with vapor-cool they yielded negative synergy.

Three different cooling methods were implemented for this study to cool small aqueous solutions. The cooling methods were: vapor-cooling or plunge-cooling into either LN or TTS. The fastest cooling method was with TTS; it cooled at a rate of 11,069 ˚C/min. The second fastest cooling method was with LN; it cooled at a rate of 1,593 ˚C/min. The slowest cooling method was vapor-cooling; it cooled at a rate of 125 ˚C/min. The required minimum concentration for the CP solution containing ethylene glycol required the same concentration at 125 ˚C/min and 1,593 ˚C/min (see Table 1-1 and Figure 1-1). Ethylene glycol was the only CP tested here that required a substantial increase in cooling rate to reduce the minimum required concentration for vitrification (see Table 1-1 or Figure 1-1). Otherwise, implementing a faster cooling method reduced each CP concentration needed for vitrification. Cooling with LN instead of vapor-cooling reduced the minimum CP concentration required for vitrification for the CPAs: 1, 2-propanediol, glycerol, isopropyl alcohol, and DMSO by 24.94 %, 22.24 %, 14.41 %, and 11.20 %, respectively. The greatest reduction of CP concentration was observed when implementing the TTS method compared to vapor-cooling. The highest reduction of a CP for vitrification was with isopropyl alcohol; it was reduced by 57.21 %. For 1, 2-propanediol, ethylene glycol, glycerol, and DMSO the reduction in required minimum vitrifiable concentration for TTS was: 50.09 %, 35.52 %, 33.28 %, and 22.24 %, respectively, compared to vapor-cooling. Overall, the molecule that was most affected when cooled more quickly was isopropyl alcohol. The difference in reduction of the percentage for isopropyl alcohol concentrations when plunge-

cooled into LN compared to TTS was 43. The difference between the percentage reduction of required minimum concentration for vitrification when plunge-cooled into LN and TTS; ethylene glycol was 35 and 1, 2-propanediol was 25. For both DMSO and glycerol, the difference between the percentage reductions for required minimum CP concentration when plunge-cooled into LN compared to TTS was 11.

Results from this study should facilitate formulation of improved CP solutions that are, in fact, capable of vitrification with the conditions used here. Further, the results from the required minimum CP concentration to achieve vitrification compared to rates of cooling point to the importance of finding even faster methods of cooling samples than what is possible with the TTS method.

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# Experiment 2

## Evaluation of similar-sized straw containers composed of different materials on rates of cooling in liquid nitrogen

(Michael J. Baker [a,b], and Charles Herr [a,b]* submitted this to the Journal for *Cryobiology*

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## Abstract

This study compared containers for cooling. Each straw type was constructed of 1 of 6 different materials. Rates were expected to follow predictions based on thermal conductivity properties or straw mass. From thermal conductivity properties, slowest to fastest would be expected to follow the order: polyvinyl chloride (PVC), polycarbonate (PC), glass (GC), quartz (QC), aluminum and silver. From straw mass data, it was expected that speed, slowest to fastest, would follow the order: PC, GC, QC, silver, PVC and aluminum. The fastest cooling rate observed was when PVC was used (4,656 ˚C/min). The second fastest cooling rate was 4,295 ˚C/min when using PC straws. Cooling rates from fast to slow were PVC, PC, aluminum, silver, QC and GC. The results were surprisingly contrary to what was expected from thermal conductivity properties, and also did not correspond with straw mass. The two materials most ideal for fast cooling rates were PVC and PC.

## Introduction

Plunge cooling is a means to create both an intracellular and extracellular ice-free state, assuming that adequate cryoprotectant concentrations are present. An ice-free state for a small volume of pure water can be achieved when it is cooled at a rate > $10^5$ ˚C/s [5]. The addition of cryoprotectants to solutions reduces the cooling rate required for vitrification [6]. However, high concentrations of cryoprotectants cause osmotic and toxic harm, which can reduce cell viability [4]. Increasing the cooling rate decreases the cryoprotectant concentration necessary to achieve an ice-free state [7]. Considerations must be made to a container's material composition and its dimensions; these characteristics influence the cooling rate [2, 7]. Our lab has an interest in straw-type containers of a particular dimension (950 µm I.D.) for plunge cooling cryopreservation. Straws of this size are convenient for use with honey bee (*Apis mellifera*) spermatozoa [3] and fruit fly (*Drosophila melanogaster*) embryos (our lab). This size straw would also be satisfactory for the storage of mammalian embryos [8]. This study focused on the effect of material composition of straws on the cooling rates in LN. The aim was to measure the cooling rates of a solution in straws made from six different materials, all of similar sizes. Choices for materials were based on their thermal properties. It was expected that straw containers constructed from thermally conductive materials would cool the medium faster than less thermally conductive materials, when plunged into LN [2]. The published thermal conductivity for polyvinyl chloride (PVC), polycarbonate (PC), patch-clamp glass capillary (GC), quartz capillary (QC), aluminum/aluminum oxide and silver are: 0.19 [7], 0.24 [9], 0.80 [2], 1.3 [7] to 8.0 [2], 250/30 [1], and 406 [2] W/m·K, respectively.

This study included straws made from six materials: PVC, PC, GC, QC, aluminum and silver. All except the aluminum and silver containers were factory fabricated in straw shapes. The silver and aluminum containers were hand-fashioned into straw shapes. The PVC straws (0.25 mL) were purchased from IMV. Both the PVC and PC (Drummond Plasticrit Plastic 75 mm

Hematocrit tubes; Cat. # 8-000-7520) straws required heat softening over a hot plate to reduce the manufacturers' dimensions to the approximate dimensions needed for this study (980 µm I.D. and 1220 µm O.D.; and, 1080 µm I.D. and 1300 µm O.D., respectively). The GC straws were purchased as a capillary tube from Warner Instrument Corp (Cat. # PG120T-7.5). The GC had the dimensions of 930 µm I.D. and 1200 µm O.D. and required no additional modifications. The QC was purchased from SP Industries (Wilmad Lab Glass; Cat. No. CV9011Q). The dimensions were 900 µm I.D. and 1100 µm O.D. and also required no additional modifications. The aluminum was purchased as a foil (Reynolds Wrap™ quality aluminum foil) and had a thickness of 17.5 µm. Because aluminum oxidizes rapidly in air, the aluminum foil was not considered to be pure aluminum. The silver was purchased as a foil with a thickness of 20.0 µm, from Arrow Springs in Cameron Park, Ca 95682-8492 (Ultra thick silver foil; 1-3/16'' X 3'). The aluminum foil and silver foil were cut from the manufacturers' roll to a length of 7 cm and a width of 0.6 cm. The 7 cm length was equivalent to the length of the other straws implemented in this study. The 0.6 cm width was chosen because it allowed each type of foil to achieve two revolutions when carefully wrapped around an 880 µm O.D. glass capillary tube (Scientific Manufacturing Industries; Cat. No.1095-A). The aluminum straw dimension was ~ 900 µm I.D and had a wall thickness of 35 µm, which was the thinnest of any of the containers in this study. The silver straw dimension was ~ 900 µm I.D. and had wall thickness of 40 µm, which was second thinnest. The fabrication process involved tightly wrapping the foil around a glass capillary and sliding the formed straw down the glass until a small interface (~ 1.25 cm) between the glass capillary and the foil straw remained. The glass capillary served as a handle during the plunging process.

Cooling rates for each material were measured ten times for each type of straw using a t-type thermocouple (Omega TT-T-40-SLE) connected to National Instruments Signal Conditioning Connector Block (SC-2345), which was connect to a DAQ™card (Al-16XE-50 e series). Acquisition

was set to measure temperature 2000 times per second. Initially, data were acquired and manipulated with Labview™, and later analyzed using Microsoft's Excel™. Straws were loaded with a cryoprotectant solution (6.2 M glycerol in DPBS) that had been visually verified to vitrify when plunged into LN. This medium eliminated the inconsistencies caused by the heat released during ice formation. The temperature range for cooling rate analysis was from 10 ˚C to -150 ˚C. Small sample inference comparing the difference between two means ($P < 0.05$) was used as the statistical test.

A precise protocol was followed when plunge cooling straws into LN. Each straw was loaded with 25 µL of 6.2 M glycerol in DPBS and sealed at the distal end with Crytoseal™. Next, temperature acquisition with Labview™ commenced at room temperature (~ 21 ˚C) and continued during cooling to LN temperature. After each cooling cycle the glycerol solution in each straw was expelled and replaced with 25 µL of fresh glycerol solution. To ensure dimensional consistency the aluminum and silver straws were re-formed around the glass capillary tube (880 µm O.D.) after each cooling cycle.

The average cooling rate for each material is presented in Figure 2-1. The PVC straw achieved the fastest cooling rate of 4,656 ˚C/min. The slowest cooling rate was with GC, which was a cooling rate of 2,001 ˚C/min. The cooling rate achieved for the materials: QC, silver, aluminum, and PC were: 2,047, 2,576, 2,783, and 4,295 ˚C/min, respectively.

The average cooling rates presented in Figure 2-1 were surprisingly inconsistent from the predictions made using the thermal conductivity values for the materials. Rates for each material relative to its mass were considered for each type of straw. The mass of the straw was measured by cutting the straws to a length equal to that containing medium during plunging.

Presented in Figure 2-2 is the mass of each type of straw and its observed cooling rate. There was a partial correlation between material mass and observed cooling rates. With GC, QC, silver, and aluminum straws, an increase in cooling rate was associated with a decrease in mass. The straws constructed from PC and PVC followed this trend only when compared with each other and not when compared to the other 4 materials. The PC straw had the greatest mass of all types of straws tested and had the second lowest published thermal conductivity and yet surprisingly, cooled its medium faster than all other types of straws, except for the PVC straws. The aluminum straw was expected to cool its solution faster than the PVC straw because the thermal conductivity of the aluminum is greater, it had the thinnest wall, and the mass of the container was less than that of the PVC straws. The results predicted from the mass and thermal conductivity properties deviated substantially from the results observed in this study. Such unexpected results could not be ignored and required an attempt for a better understanding. The possibility of the PVC and PC straws being permeable to the passage of LN and therefore being somewhat transparent to the cooling fluid was considered. The different types of straws were tested for LN permeability and were compared to cooling rate data.

Five types of straws were assayed for LN permeability. The types of straws tested were GC, PC, PVC, silver and aluminum. Permeability was tested for GC, PC and PVC straws by heat-softening un-stretched straws and shaping them into a “U”. This approach eliminated the need for heat-sealing or the use of Crytoseal™, so as to eliminate the risk of LN leakage through an improper or failed seal. After forming the straws into a “U” shape; the “U” portion was submerged into LN for 10 to 12 min. The degree of LN permeability was determined by visual observations. The aluminum and silver straws were sealed by metal forceps, which were used to crimp 0.1 cm of the straws’ ends. The crimped end was then folded it over-itself. Crimp and folding was repeated 2 additional times. The aluminum and silver straws were submerged into

LN for 3 to 4 s. Upon removal from LN, the glass capillary handle was promptly positioned at the opening of the technician's ear canal. This approach made it possible to hear and feel LN vaporize. There was evidence for entry of LN into the metal straws. However, LN had to travel 0.6 cm along the inner side of both foil straws to reach their inner medium. A scale from 0 to 10 was used to score the degree of each straw's permeability to LN. If a straw was permeable through direct visual observation, audio observation or impermeable to LN a score of 10, 5, or 0 was assigned, respectively.

Presented in Figure 2-3 is the degree of LN permeability presumed for the aluminum and silver through audio observations, and observed for GC, PC, and PVC straws. The PVC straw was assigned a 10 for LN permeability. Liquid nitrogen permeated the PVC straw and had accumulated to a volume easily visible. It was presumed that the noise heard from the bursts of vaporized liquid, which had impacted the technician's ear canal, were an indication that LN had leaked into the foil straws, so these were assigned a 5. The porosity of the PVC straws might have had a substantial effect on cooling rate (Figure 2-1). Liquid nitrogen permeability of PC straws was not observed after submerged in LN for 10-12 min. The potential for accumulation of condensed ambient atmospheric gas into un-sealed PC and un-sealed GC straws when submerged in LN for 4 hrs was a concern. Therefore, each of these PC and GC straws had its end heat-softened with a Bunsen burner and was then folded-over against it-self and crimped with heated metal forceps to a create heat-seal. The PC straws required 4 hrs of submergence in LN to accumulate fluid, although this observation was inconsistent. No visible volume of LN accumulated in GC straws after being submerged in LN for 4 hrs.

In this study, our primary objective was to identify the straw material that would provide the fastest rate of cooling among the 6 different materials tested. Of secondary consideration

were the practical properties of these materials. The cost for the QC straws was the highest compared to the other 5 materials used in this study. In addition, the modification of quartz straws would require the use of oxy-acetylene equipment. The materials that produced the fastest rate of cooling were the least expensive, most convenient to modify, and also were transparent. These materials were PVC and PC. Although an understanding for why these materials provided the fastest rates of cooling is inadequate, permeability to LN might be a factor.

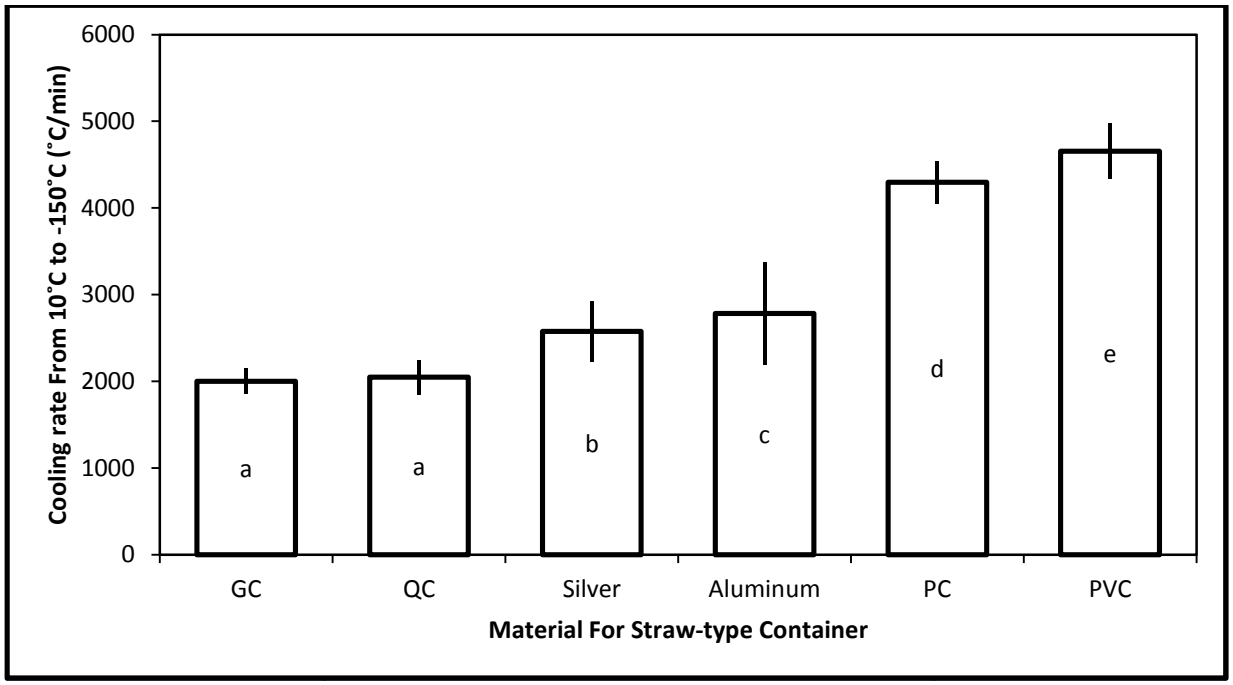


**Figure 2-1. Cooling rate averages from ten replicates of plunge cooled patch glass (GC), quartz (QC), silver, aluminum, polycarbonate (PC), and polyvinyl chloride (PVC) straws into liquid nitrogen. Error bars represent standard deviation. Bars with different letters are significantly different ($P < 0.05$).**

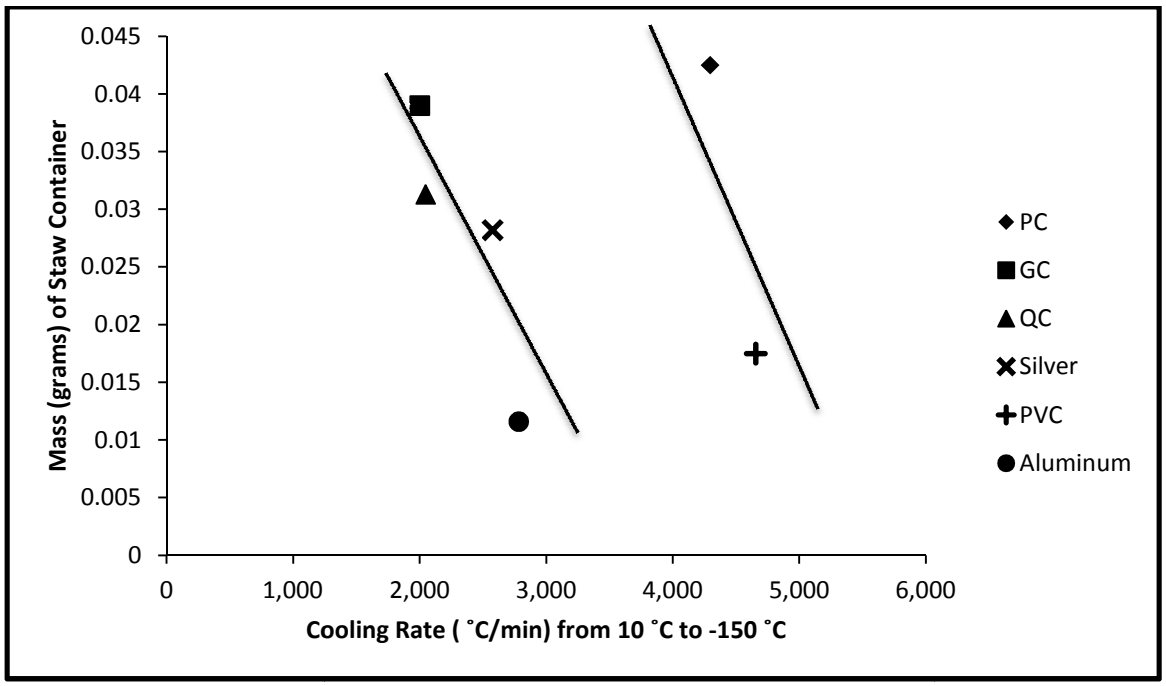

**Figure 2-2. Material mass for each straw container compared against their respective cooling rate average.**

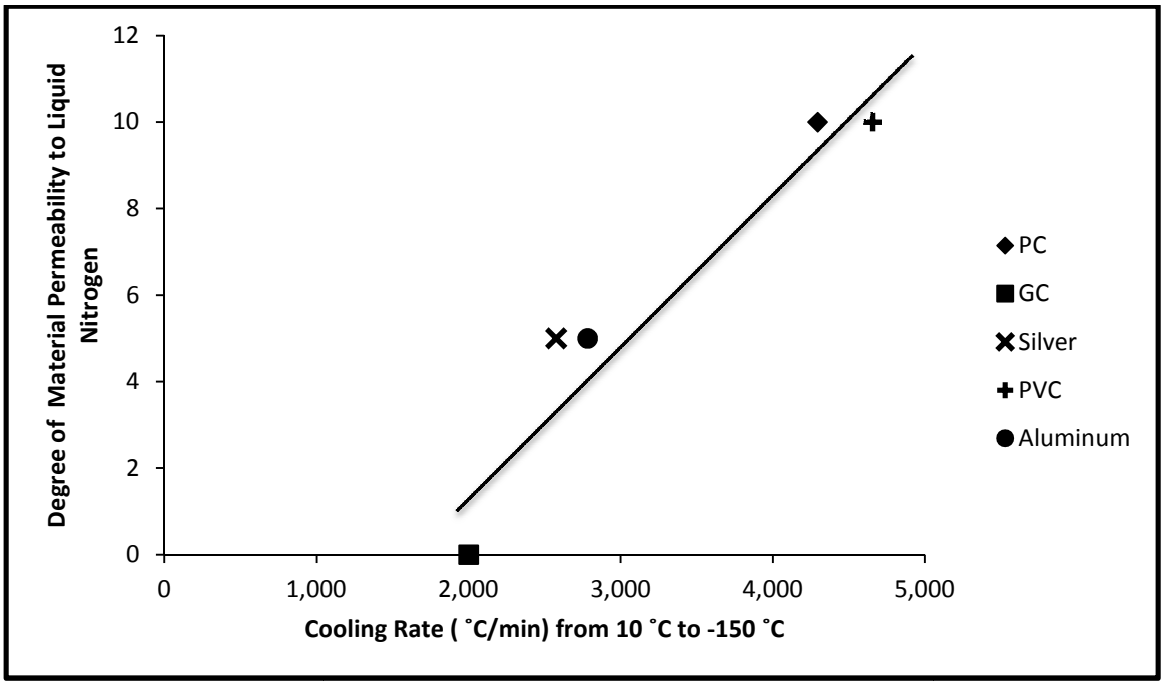


**Figure 2-3. Liquid nitrogen permeability based on direct visual and audio observations for five materials composed as straw containers, compared against their respective cooling rates.**

**Acknowledgement**

We would like to thank Brandon Hopkins for his editorial comments.

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# Experiment 3

## An explanation of why it is difficult to form slush nitrogen from liquid nitrogen that had been previously used for this purpose

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## Abstract

Slush nitrogen (SN) is used to avoid the Leidenfrost effect, which is problematic when using liquid nitrogen (LN). Slush nitrogen's usefulness has been demonstrated by its requirement for the successful cryopreservation of insect embryos. To convert LN to SN, typically, the pressure above a Dewar of LN is reduced, using a vacuum pump in a sealed system until conversion occurs. It has been observed that LN from a fresh tank will readily produce SN; however, repeated use of the same LN results in the inability to form SN in subsequent trials. The current experiments were designed to identify the cause of this phenomenon. The hypothesis is that oxygen (g) from the surrounding, ambient air condenses and mixes with the

LN to form a mixture with a lower freezing point and; therefore, prevents the formation of SN. The hypothesis was tested and was verified.

## Introduction

In regards to cooling rates, there are two general approaches to cryopreserving cells. The first is called slow-equilibrium freezing. An example of a slow-equilibrium freezing protocol is when cells are cooled from 0 ˚C to -40 ˚C at < 3 ˚C/min and then plunged into liquid nitrogen (LN) for storage. The disadvantages of slow-equilibrium freezing for some cell types are their sensitivity to the pressures of extracellular ice formation (EIF) [1, 3] and chilling injury [2, 9]. These issues cause failure in the form of cellular death. Another approach to cryopreservation, the focus here, is termed vitrification. Vitrification reduces problems caused by EIF and chill injury. Vitrification attempts to create an amorphous solid state, extracellularly and intracellularly. Typically, cells are rapidly cooled from room temperature to -196 ˚C by plunging the container containing the cells into LN. A countering effect on achieving speed when cooling with LN is the formation of a vapor layer around the plunged specimen. The warmer specimen vaporizes the LN and the vapor insulates the specimen; an event referred to as the Leidenfrost effect [6]. The Leidenfrost effect is most commonly described as the phenomenon in which a liquid, in near contact with a mass significantly hotter than the liquid's boiling point, produces an insulating vapor layer which keeps that liquid from boiling rapidly. In the case of rapid freezing, the insulating vapor layer also prevents the solid mass from rapid cooling, a phenomenon that must be avoided in cryopreservation. Reduction in the Leidenfrost effect can be achieved using a semi-solid form of nitrogen called slush nitrogen (SN). In 1960, Luyet and Kroener [7] found the cooling rate with SN was substantially faster to LN. Slush nitrogen has been used to cryopreserve *Drosophila melanogaster* embryos [8] and mammalian embryos [5].

Over a 2.5 yr period, our lab has had predictable, but unexplainable, difficulties in making SN. Slush nitrogen could be readily produced with a fresh batch of LN. When the LN had been used several times prior to making SN, it became difficult to impossible to get the LN into a semisolid state. We discarded a theory that LN had "memory", like the Mpemba effect of water [4]. Efforts to find an explanation for this observation included scrutiny of our home-made vacuum chamber and other components used for making SN. Even after holding LN in the vacuum chamber for nearly four times as long as required to succeed with a fresh tank of LN, SN failed to form when using LN that had been previously taken through the freezing cycle several times. Next, the possibility of contamination with other dissolved gasses was explored.

Earth's atmosphere consists of approximately 79 % nitrogen, 20 % oxygen and a small concentration of other gasses. It seemed possible that oxygen, the second most abundant gas, might have condensed into the slush nitrogen. Liquid nitrogen has a lower boiling point (-196 °C) than liquid oxygen (LO, -183 °C) and vessels containing liquid nitrogen can condense oxygen from the surrounding, ambient air. Our hypothesis is that a mixture of LN and LO is unable to form a slush under the conditions of the experiments. In this study, aliquots taken from a fresh tank of LN and from LN that had been previously taken through several cycles of slush formation and were subsequently "non-slushable" were analyzed for oxygen content.

The percentage of oxygen in LN was quantified using a Vernier Labquest (serial #1003893) hand-held monitor and Vernier oxygen gas sensor (02-BTA). A 250 mL Nalgene™ bottle, which had been pre-chilled to LN temperatures, was filled with 100 mL aliquots of LN. Immediately following the addition of the 100 mL aliquots; a sequence of deflated latex balloons (Gayla Industries, stock # 30901) were placed around the opening of the bottle. The latex balloons captured the gaseous molecules from the boiling liquid. Typically, five balloons were required to

capture the gas phase of a 100 mL liquid volume. After filling each balloon with gas, a hemostat was used to close the balloons until their attachment to the oxygen sensor. The diameter of the oxygen sensor (2.8 cm) allowed for trouble-free attachment of the balloons. The balloons' elasticity ensured positive pressure against the wall of the oxygen sensor. Once the balloon was attached to the oxygen sensor, a final room oxygen measurement was recorded and then the hemostat was relaxed, allowing the contents of the balloon to escape through small holes on the oxygen sensor's housing. This method provided a steady stream of gaseous molecules from the balloons for accurate oxygen concentration measurements. Oxygen measurements were recorded for each balloon every 60 s. A typical balloon expelled its contents through the oxygen sensor in less than 240 s.

Oxygen concentrations were estimated from the means of the 60 s and 120 s interval measurements for each balloon within a treatment. These time points were chosen because a constant stream of gas was present then, which was necessary for accurate measurements. It was observed that when the balloons became flaccid, the oxygen sensor showed higher oxygen readings, which was expected.

Four 100 mL aliquots of LN from a typical laboratory tank (34 L Taylor-Wharton), which held LN that had been repeatedly used to make SN, enough so the LN would no longer form SN, were assayed for LO. The first and second aliquots were examined by capturing the gas of the boiling LN in series from balloons labeled one through five. Oxygen concentrations were then measured in the same order. The third and forth 100 mL aliquots were performed by placing the Nalgene™ bottle in a cardboard box (length 32 cm, height 35 cm and width 26.5 cm) that had been purged with argon. The height of the argon was determined by slowly lowering a flame from a Bic™ lighter into the box until the flame was extinguished. This approach ensured

the Nalgene™ bottle was isolated from atmospheric oxygen. Boiled LN was captured and measured for oxygen concentration in a series identical to the first and second aliquots.

We measured oxygen concentration from unenriched LN obtained from a full 18 L LN laboratory tank. It was noted that this LN tank, prior to it being filled with fresh LN, was acclimated to room temperature. From this, two 100 mL aliquots of LN were measured for oxygen concentration in corresponding order of fill.

LN was enriched with oxygen by two methods. Both methods involved placing the opening of a PVC tube (approx. 3 mm O.D.) from an oxygen source to 7 cm below the surface of a container of LN. A fine stream of oxygen bubbles served to introduce the LO into the LN. In the first method, gaseous oxygen was bubbled into 1 L of LN for 8 min and 100 mL of this was assayed for oxygen. The second method streamed oxygen gas for 12 min into 2.1 L of LN, of this, 1.6 L was used in a failed attempt to make SN, and then 100 mL of this was assayed for oxygen concentration.

Visual comparisons were made between 1.6 L of unenriched LN and oxygen enriched LN during SN production. Unenriched LN or LN enriched with oxygen (obtained from the second method described above for enriching fresh LN with oxygen) was placed into a Dewar (15 cm I.D., 17 cm O.D., 33 cm height) and then placed into a vacuum chamber. A Hitachi vacuum pump (model # N9028) reduced the pressure in the vacuum chamber to 584.2 torr. A subjective criterion for the SN formed was its solid appearance, which was observable through a Plexiglas™ window on the top of the vacuum chamber. A scale of 0 to 10 was assigned to each trial; a score of zero was given when no SN was present. If the SN easily dissipated after being physically disturbed it was scored a one. If the SN was not dissipated after being disturbed, it was scored a ten. The integrity test was performed using physical disturbances in two ways:

first, hitting the top of the vacuum chamber with a closed fist and second, tilting the vacuum chamber onto one side and letting it suddenly drop onto the Styrofoam™ pads. The Styrofoam™ pads primarily served to isolate the bottom of the vacuum chamber from the floor of the lab.

The highest percent of oxygen was found in the last balloon filled of each aliquot (Figure 3-1 and Figure 3-2). The lowest percent of oxygen was found in the first balloon filled of each aliquot (Figure 3-1 and Figure 3-2), except for aliquot 1 in Figure 3-1. In aliquot 1 (Figure 3-1) the second balloon filled contained the lowest percent of oxygen at 1.34 %, whereas the first balloon filled in aliquot 1 (Figure 3-1) contained the second lowest percent of oxygen at 1.55 %. The sixth balloon in aliquot 4 (Figure 3-1) captured the last remaining portion of boiling liquid (~ 7 mL) from the Nalgene™ bottle, which had purposely not been captured with the five balloons in aliquot 3 (Figure 3-1). This demonstrated LO propensity to remain liquid until the LN has evaporated. An explanation for this is the fact that LO has a higher boiling point than LN. For each aliquot, as the 100 mL LN decreased in the Nalgene™ bottle, an increased percent of oxygen was found.

The integrity of SN was greatest when using fresh LN (Figure 3-3). Fresh LN took approximately 10 min to commence formation of SN. Whereas, fresh LN enriched with oxygen took approximately 13 min to commence formation of SN. At 14 min, fresh LN took the form of a thick opaque solid, whereas fresh LN enriched with oxygen struggled to transition into SN. Fresh LN enriched with oxygen was observed for 14 min after commencing formation of SN. During this 14 min, no significant amount of SN formed and what did form was easily destroyed when physically disturbed. All attempts to disrupt the SN formed from fresh LN failed (i.e., the SN was stable). The integrity score for SN formed from using fresh LN was a ten and fresh LN enriched with oxygen was a one.

The repeated use of LN from a 34 L holding tank resulted in an oxygen concentration that limited the ability to make SN. Using LN enriched with oxygen mimics what was observed with our repeated use of the same LN. Using oxygen adulterated LN in a vacuum chamber will only transition into a thin, scarce floating layer of SN. The SN formed was not stable and was easily destroyed by minimal perturbations. Increasing the time in the vacuum chamber had no positive influence toward creating SN when using LN enriched with oxygen. We found the use of pure LN was essential to making SN.

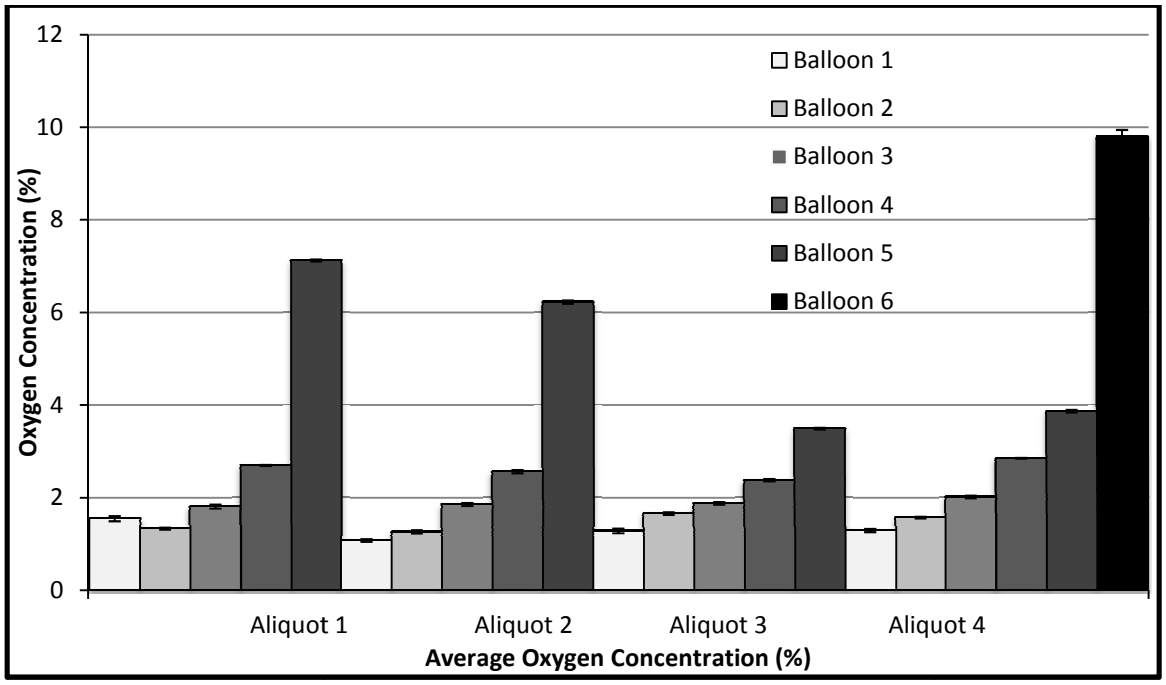


**Figure 3-1. Average percent oxygen concentration from the suspected $O_2$ contaminated LN tank for four aliquots consisting of five to six balloons each. Aliquots 1 and 2 are from the balloons filled and measured in sequence for $O_2$, from balloons 1 through 5. Aliquots 3 and 4 are from shielding the Nalgene bottle with argon gas and assayed same as Aliquots 1 and 2. Error bars represent standard deviation for oxygen measurements at 60 s and 120 s.**

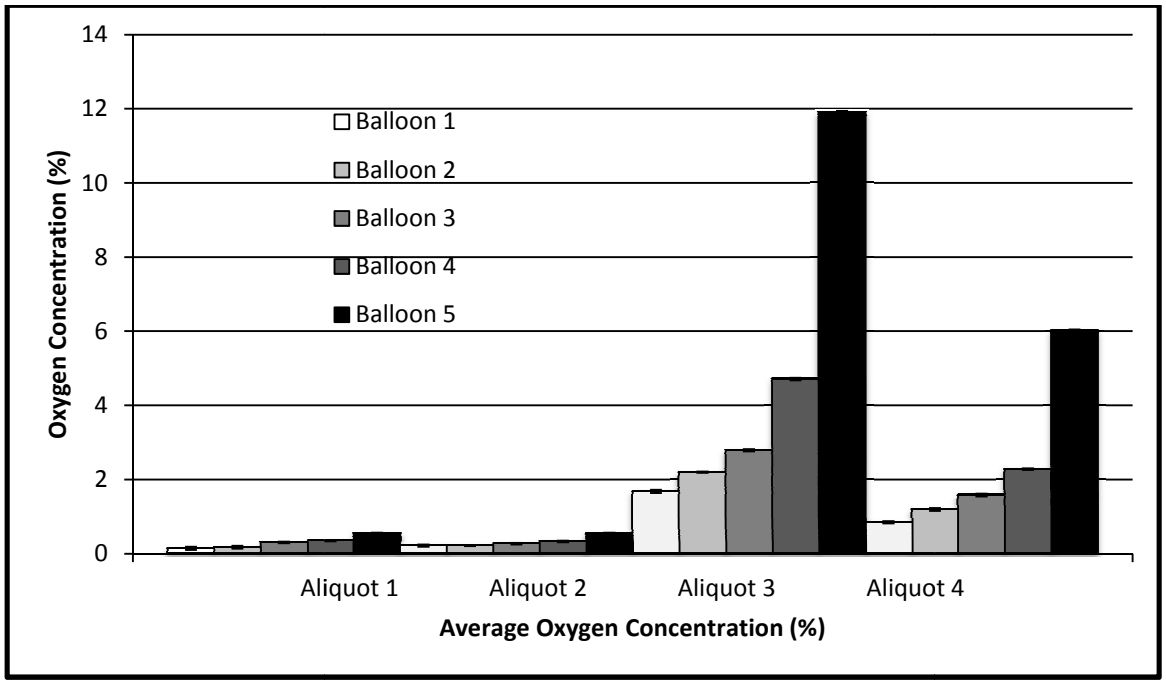


**Figure 3-2. Average percent oxygen concentration from fresh LN and fresh LN enriched with oxygen for four aliquots consisting of five balloons each. Aliquots 1 and 2 are from fresh LN. Aliquot 3 is from 1 L fresh LN enriched with oxygen for 8 min. Aliquot 4 is from fresh LN enriched with oxygen for 12 min. Of this, 1.6 L was used in a failed attempt to make SN. Error bars represent standard deviation for oxygen measurements at 60 s and 120 s.**

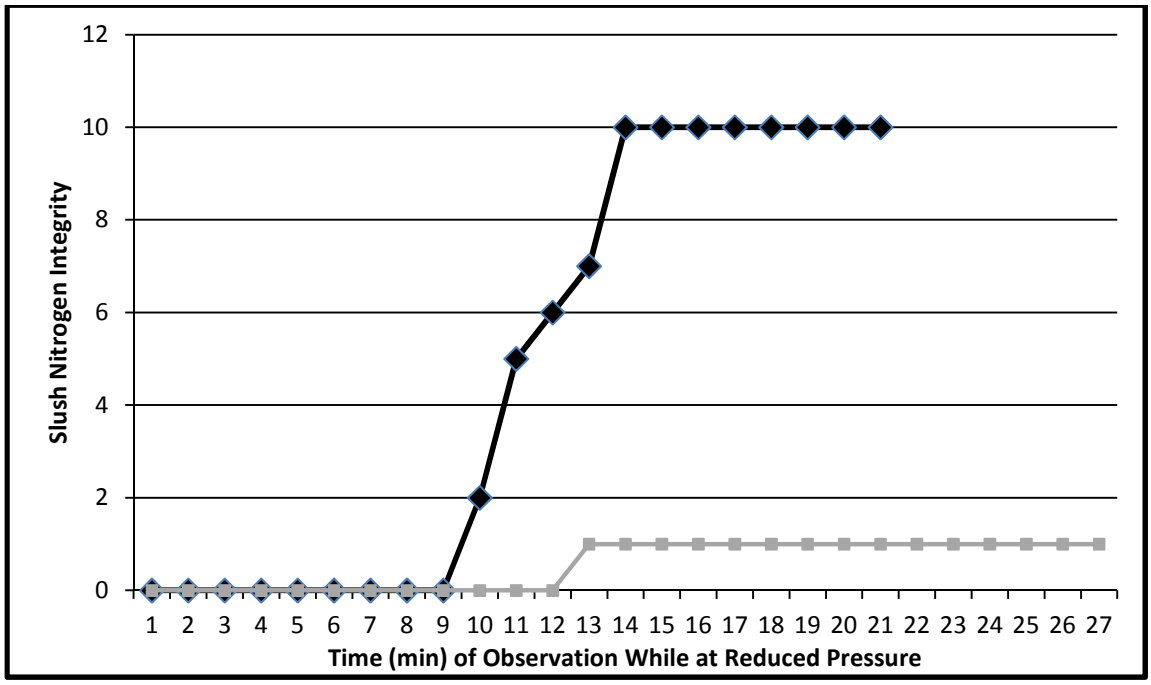


**Figure 3-3. Slush nitrogen integrity vs. time (min) of visual observations while making SN from 1.6 L of unenriched LN (diamond) and 1.6 L of LN enriched with oxygen (squares).**

Acknowledgements

We are grateful to those who donated funds to CANBE and we would like to thank Dr Jason Acker for technical assistance of this manuscript.

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# Experiment 4

## Two novel methods for accelerating the cooling of small volume samples

(*Michael J. Baker [a] and Charles Herr [b] have had this abstract published in the Journal for *Cryobiology*

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*A Presentation for The 48th Annual Meeting of the Society for Cryobiology, Corvallis, Or 2011)*

## Abstract

When sufficiently rapid cooling occurs, water will solidify, ice-free, creating a vitrified state. The rate required has been estimated to be $10^8$ ˚C/min. Volumes needed to contain cells are generally too large to achieve this. Addition of cryoprotectants (CPs) is required if vitrification is to occur at slower rates.

Two important features when plunging into liquid nitrogen (LN) or slush nitrogen (SN) are rates of cooling and uniformity of rates between sample plunges. How fast a sample is cooled to below the glass transition temperature influences the concentration of CP required to vitrify. Since higher concentrations of CPs have higher toxicity, accelerating the rate of freezing above what has been possible might have advantages. A second factor is how uniform and therefore predictable the cooling rates are between plunges. Knowledge of the uniformity of cooling rates might enable more accurate estimation of the needed CP concentrations, as well as help generate more predictability.

Cooling rates were measured for samples plunged into LN or SN. Also, two novel methods were investigated: liquid nitrogen vortex (LNV) and turntable slush (TTS). The LNV method used a magnetic stir bar to create a spinning vortex of LN. The TTS method was accomplished by turning a container of 1600 ml of SN at 45 rpm. Mean cooling rates were calculated from the measurements of 20 plunges for each of the cooling methods.

Measurements were made using a 75 µm T-type thermocouple (Omega TT-T-40-SLE) connected to a National Instruments Signal Conditioning board (SC-2345), which was connected to a DAQ™card (AI-16XE-50 e series). Data were acquired and initially manipulated using Labview software. Data were then transferred to Microsoft Excel for further analysis. Presented here are the results for the temperatures ranging from 10 to -150˚C. Quartz capillary tubes (1.5 mm ID and 0.26 mm wall thickness) containing 25 µl of water were plunged into the coolant with considerable effort concentrated on the speed of the plunge. The fastest mean rate of cooling was achieved with TTS (11,069˚C/min). Cooling rates for SN, LNV and LN were 8,424, 2,715 and 1,593 ˚C/min, respectively. Standard deviation values were normalized to the slowest cooling rate: LN. The standard deviations were 48.6, 116.4, 247.1 and 429 for LN, LNV, TTS and SN. The cooling rates, when turning SN at 78 rpm were 9,919˚C/min, slower than the speed achieved by spinning at 45 rpm. Turning TTS at 33 rpm, although not quantified, failed to vitrify a minimum CP concentration that did vitrify using TTS rotating at 45 rpm. The fastest mean cooling rate was achieved with TTS at 45 rpm. The TTS cooling method also had a lower standard deviation than SN. Rates in TTS rotating at 45 rpm were 7 times faster than using LN, and 2,645˚C/min faster than SN. The LNV method yielded a faster mean cooling rate than LN. LN provided the lowest standard deviation and therefore greatest uniformity, but was substantially slower. The TTS method had clear advantages over SN in being more uniform between plunges and faster.

## Introduction

The transition from ambient room temperature to liquid nitrogen (LN) temperature for cryopreservation can be performed in two ways. One way is slow-equilibrium freezing. It can be described as a two part process; first involves cooling a sample from 0 ˚C to -40 ˚C at a rate of 1˚C to 3 ˚C/min and second the rapid cooling from -40 ˚C to -196 ˚C. One disadvantage to this method is that it subjects cells to lethal ice pressures (Fujikawa and Miura, 1986). In addition, many cells types are sensitive upon cooling to 0 ˚C and below (Collins and Mazur, 2006; Mazur, Schnieder and Mahowald, 1992). The alternative method was developed for cells that are unable to transition this critical temperature region. The alternative method subjects cells to a rapidly fast cooling rate to outrace water crystallization to create an amorphous non-crystalline state. The amorphous state is commonly referred as *vitrified*. It is achieved when a sample is rapidly cooled by liquid nitrogen (LN) or slush nitrogen (SN) to below the glass transition temperature. The glass transition temperature is defined at a point when a material has reached a viscosity of $10^{13}$ Poise, for water this is at -136 ˚C. Vitrification of water is estimated to require a cooling rate of $10^{8}$ ˚C/min. The use of a cryoprotectant (CP) reduces the cooling rate necessary for vitrification (Rall and Fahy, 1985). Currently, the cooling medium that can achieve the fastest rates is SN. A faster cooling rate will reduce CP concentrations. However, the required minimum CP concentration for vitrification is still toxic for most cell types. This project attempted to accelerate the cooling rates that are currently possible with SN. Another variable of concern is the predictability of freezing rates using different cooling media and variation between samples.

We suspect reducing variation between cooling rates with SN by rotating it on a turntable will reduce CP concentrations to less toxic levels. In this study, we quantified cooling rates for LN, SN and two novel methods of cooling; liquid nitrogen vortex and turntable slush. It is our

thought for the cooling methods LNV and TTS an accelerated cooling rate will be quantified and a reduced variation between cooling rates observed for the same sample.

## Materials and Methods

Cooling rates were quantified for 25 µl of water contained in a quartz capillary tube (1.5 mm inner diameter and 0.26 mm wall thickness) using a 75 µm diameter t-type thermocouple (Omega TT-T-40-SLE) seen in Figure 4-1. The thermocouple was attached to a National Instruments signal conditioning board (SC-2345; see Figure 4-1) interfaced through National Instruments DAQ™card (AI-16XE-50 e series). National Instruments Labview software collected the temperature measurements at 2000/s and simultaneously stored them to a computer hard-drive on a desktop computer. This allowed for later analysis of data via Microsoft Excel. Twenty replicates were performed for each cooling medium. Each sample was plunged to below the surface of their respective cooling medium. When plunging samples into the rotating cooling media the quartz was a distance of 0.1 to 1.0 cm from the inner container wall.

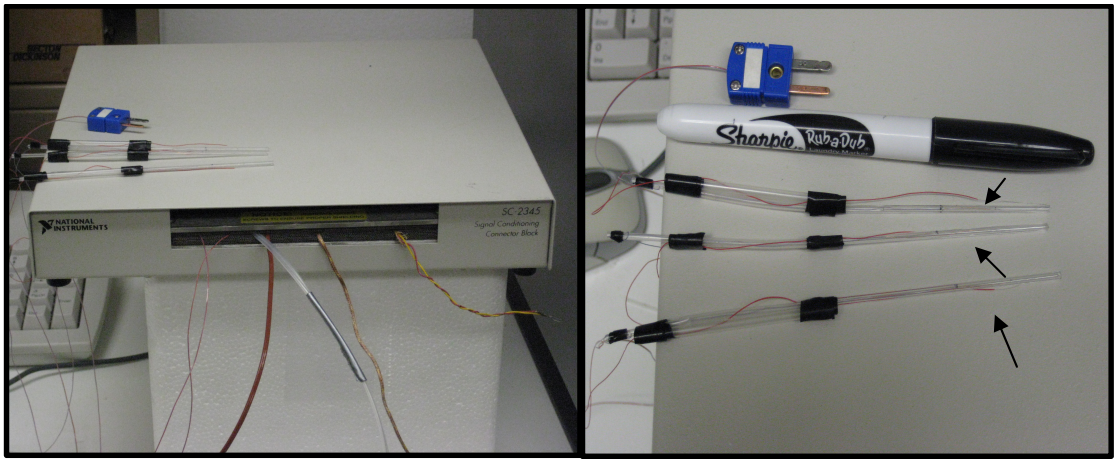


**Figure 4-1 - National Instruments Signal Conditioning Board (left-hand side) with attached t-type thermocouples that have been inserted into a quartz capillary tube. The right-hand side is the quartz capillary tubes which are attached to a polyvinyl**

**chloride (PVC) insemination straw with black electrical tape. The black arrows depict the location for each junction of the t-type thermocouples.**

An expanded polyvinyl chloride container (U.S. patent #2,707,503) as can be seen in Figure 4-2 (18 cm height, 20 cm top inner diameter, 14.5 cm bottom inner diameter and 1.5 cm wall thickness) contained the cooling medium. The volume of each cooling medium was different. For LN and LNV the container was filled with 3 L of LN. The LNV method (see Figure 4-2) was executed using a magnetic stir bar (7.5 cm length and 1.25 cm diameter) placed in the container of LN spun at a speed rating at 9.25 of 10 (Fisher Jumbo magnetic stirrer by Fisher Scientific Co.).


**Figure 4-2 - Magnetic stirrer used to spin a magnetic stir bar that was contained in a polyvinyl chloride container filled with liquid nitrogen to produce vortex of liquid nitrogen.**

Our home-made vacuum chamber (Figure 4-3) contained 1.6 L of LN and was reduced to 23 inches Hg vacuum (Hitachi 160VP N9028) to create SN. The process took between 10 to 30 minutes. Rotating SN at 45 rpm (General Electric 11-4022B) with a turntable seen in Figure 4-3 created TTS effect. Quantification of cooling rates for both SN and TTS was performed 2 to 5 minutes post removal from vacuum. This ensured consistent milk-shake like SN. Rotational SN at 78rpm (Soundesign S-3206; see Figure 4-4) was performed 10 times. Quantified cooling rates were averaged and normalized to the slowest cooling rate (LN) for pair-wise comparisons. Statistical analyses of the normalized cooling rates were performed by test statistics ($P < 0.05$) using small sample inferences concerning the differences between two means. The quantified cooling rates were from the temperature range 10 ˚C to -150 ˚C.

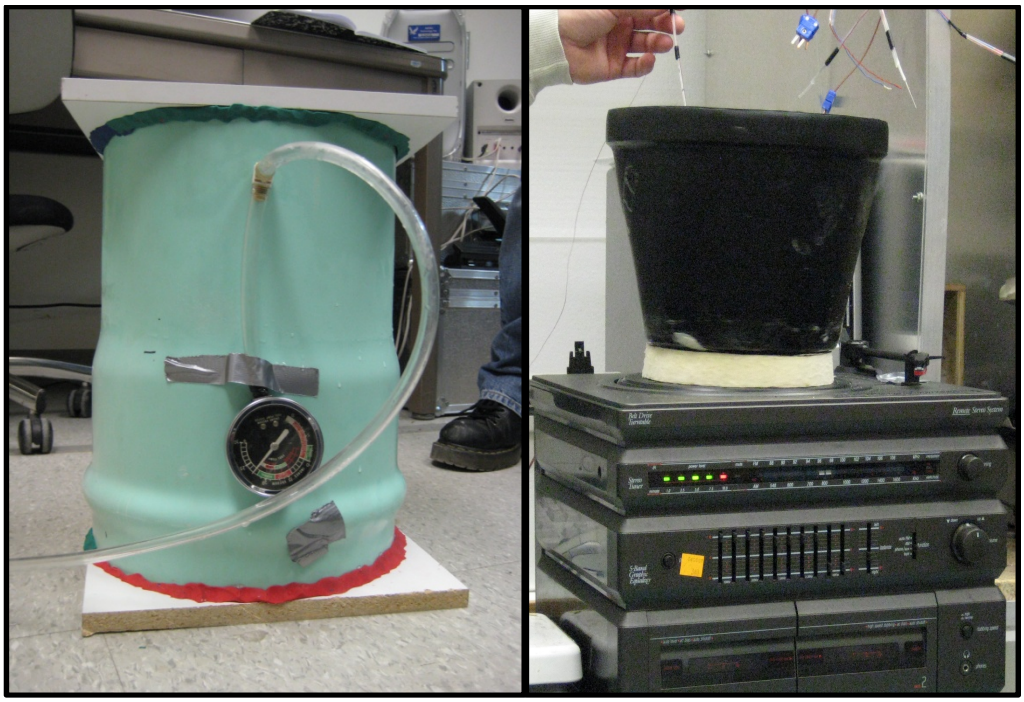

**Figure 4-3 - Home-made vacuum chamber used to make slush nitrogen from liquid nitrogen (right-hand side). On the left-hand side is the turntable used to create the turntable effect by rotating slush nitrogen at 45 rpm for turntable slush. For video of**

**the research team using the vacuum chamber, and the thermocouple construction, go to: http://www.youtube.com/watch?v=mlC_HKtgBiE&feature=related.**

**Figure 4-4 - Turntable used to create the turntable effect by rotating slush nitrogen at 78 rpm for turntable slush.**

**Results**

Figure 4-5 shows a summary of mean cooling rates for 25 µl of water plunged into 4 different cooling media from 10 ˚C to -150 ˚C. The TTS method achieved the fastest mean cooling rate of 11,069 ˚C/min. Cooling rates for SN, LNV and LN were 8,424; 2,715 and 1,593 ˚C/min, respectively. The standard deviation values normalized to the slowest cooling rate were 48.6, 116.4, 247.1 and 429 for LN, LNV, TTS and SN, respectively. The standard deviation normalized values are significantly different ($P < 0.05$). Not included in Figure 4-5, the mean cooling rate for rotating SN at 78 rpm was 9,919 ˚C/min. Figure 3 shows the thermal history of 25 µl of water plunged into 4 different cooling media from 10 ˚C to -150 ˚C. The purple line (Figure 4-6) depicts the thermal history for the TTS method when cooling 25 µl of water. The

green, red and blue lines are for the thermal history of SN, LNV and LN cooling methods, respectively.

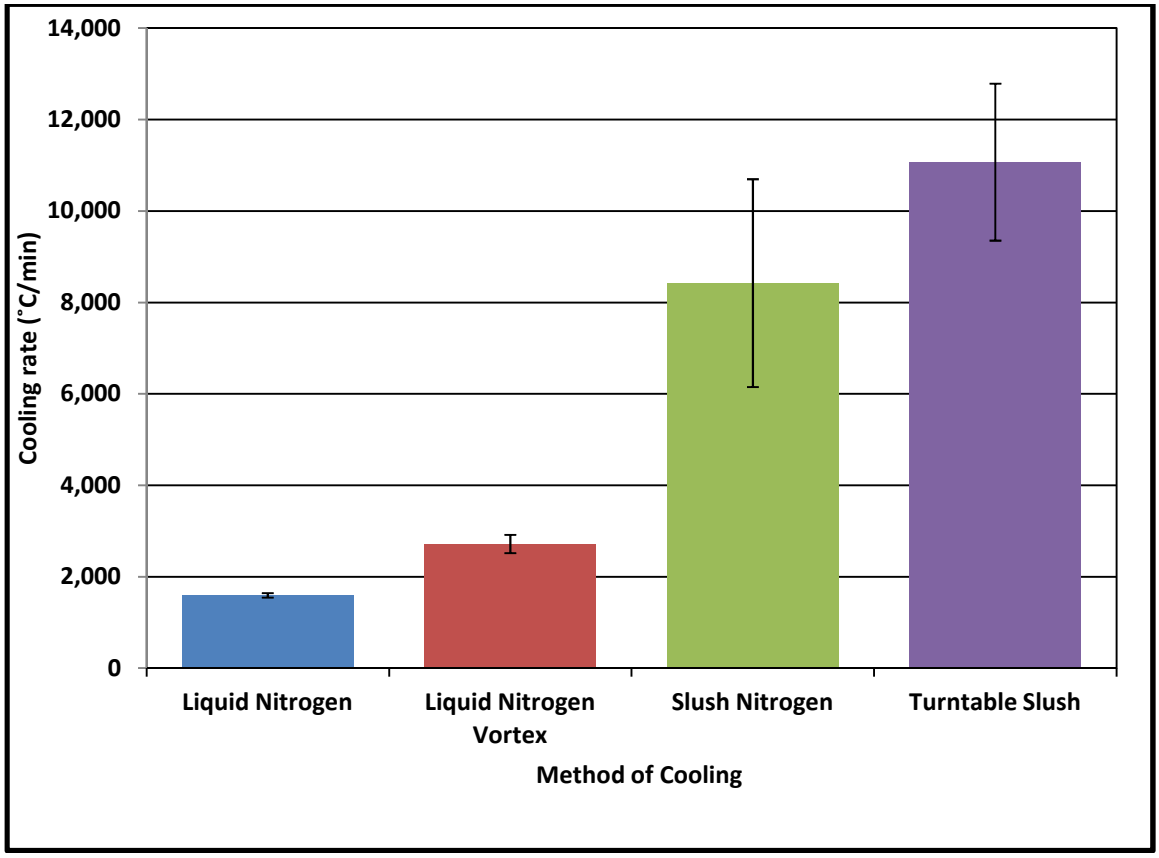


**Figure 4-5 - Cooling rates for quenching 25 µl of water contained in quartz capillary. Cooling rates are from 10 ˚C to -150 ˚C.**

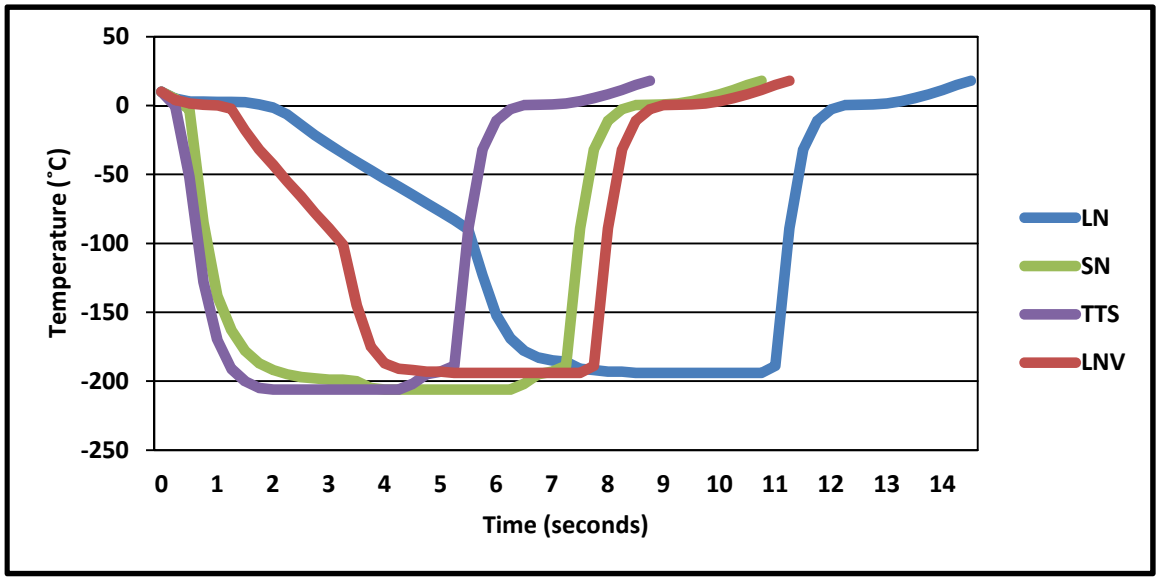


**Figure 4-6 - Thermal history summary of the four methods of cooling of 25 µl of water contained in a quartz capillary.**

**Discussion**

Accelerated rates of cooling were achieved with SN. However, using SN might be detrimental to cells, possibly caused by variation in cooling rates between plunges (Risco *et al.*, 2007). Our lab has reported two novel methods for accelerating the rate of cooling for small volume samples. The fastest mean rate of cooling was achieved with SN rotating at 45 rpm. An additional benefit from rotating SN at 45 rpm was a more predictable cooling rate compared to SN. The method of cooling that was the most predictable was LN, but it achieved the slowest mean cooling rate. The SN method when rotating at 78 rpm achieved a mean cooling rate 9,919 ˚C/min and was slower than rotating SN at 45 rpm. The cooling method, SN at 78 rpm was limited to 10 replicates because initial quantified mean cooling rates were slower than rotating SN at 45 rpm and generated greater standard deviation than SN (586.5). Although rotating SN at 33 rpm was not quantified, this method was unable to vitrify a solution that was vitrifiable

when rotating SN at 45 rpm. The LNV method has a clear advantage over LN in achieving faster cooling rates. However, it has the disadvantage of having a greater standard deviation than the LN method. The advantages of TTS are clear — it will cool faster and more uniformed than SN. These characteristics of TTS can potentially contribute to the formulation of accurate CP concentrations for vitrification of small volume samples.

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# Vita

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## Education

| | |
|---|---|
| Associate of Arts and Science | 2005 Olympic College, Bremerton, Washington |
| Bachelor of Science (Biology) | 2008 Eastern Washington University, Cheney, Washington |
| Master of Science (Biology) | 2012 Eastern Washington University, Cheney, Washington |

## Professional Work Experience

| | |
|---|---|
| 2009 | Eastern Washington University, Cheney, Washington<br>Teaching assistant for General Biology |

## Volunteer Work

| | |
|---|---|
| 2008 to present | Center for Animals Near Biological Extinction (CANBE) |
| 2010 | Science Olympiad State Competition<br>Proctored and evaluated Cell Biology event |

## Research Focus and Activities at Eastern Washington University

### Interests:

**Developing relevant cryopreservation protocols for preservation of endangered species**
**Understanding the prevention of ice-formation for relevance to cryopreservation**

### Activities:

**Micromanipulation**
**Designing cryopreservation apparatus'**
**Cryopreservation of *Drosophila melanogaster embryos***
**Design and construction of novel cryopreservation cooling procedures**

## Relevant Science Courses

1. **General Chemistry (1 year)**
2. **Organic Chemistry (1 year)**
3. **Biochemistry (2 quarters)**
4. **Microbiology**
5. **Advanced Microbiology**
6. **Immunology**
7. **Epidemiology**
8. **Hematology**
9. **Histology**
10. **Cell Biology**
11. **Genetics**
12. **Physics (1 year)**
13. **Calculus**
14. **Vertebrate Zoology**
15. **General Biology (1 year)**

## Referred Publications

1. Baker, M.J., and Herr, C. Two novel methods for accelerating the cooling of small volume samples. Cryobiology. 2011; 63: 333-334.

## Manuscripts Accepted for Publication

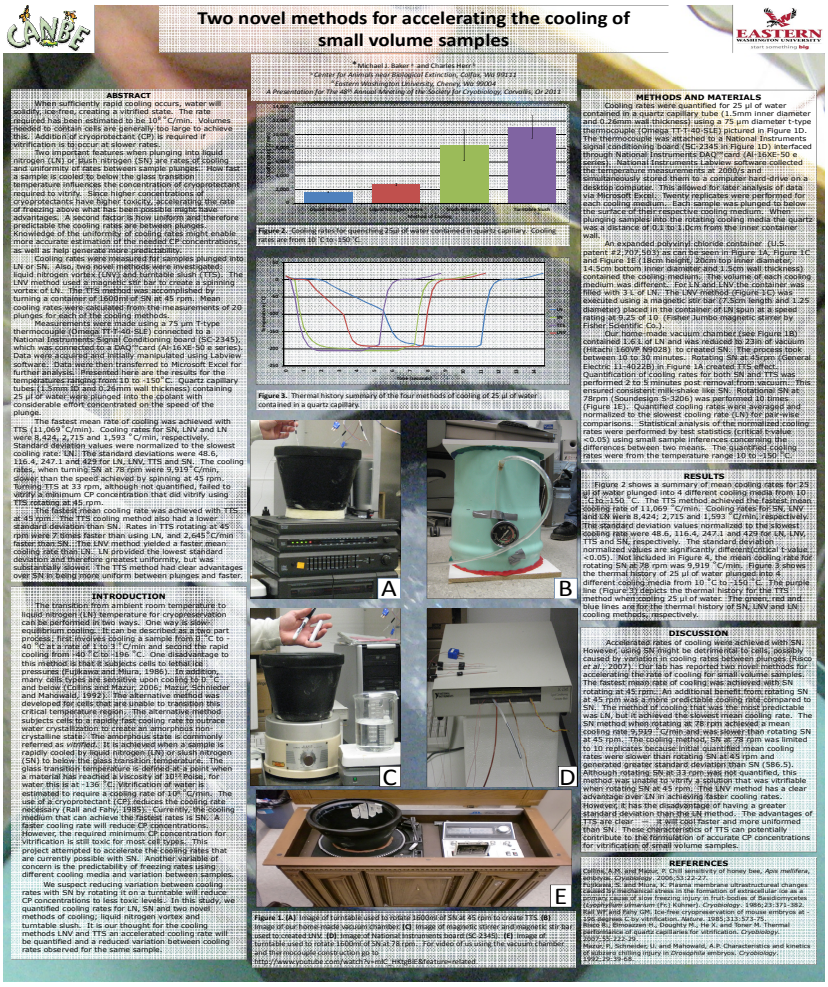
1. Baker, M.J., Denton, T.T. and Herr, C. An explanation for why it is difficult to form slush nitrogen from liquid nitrogen used previously for this purpose. Accepted to the *Journal for Cryobiology.*

## Manuscripts Currently in Review for Referred Publication

1. Baker, M.J., and Herr, C. Evaluation of similar sized straw containers composed of different materials on rates of cooling in liquid nitrogen. In review for the *Journal for Cryobiology.*

## Presentations

2011 Baker, M.J. and Herr, C. Two novel methods for accelerating the cooling of small volume samples, *A Presentation for The 48th Annual Meeting of the Society for Cryobiology, Corvallis, Or* (Poster presentation -- shown below)




2009 Baker, M.J., and Herr, C. Quantifying the Rates Achievable Using Different Methods of Liquid Nitrogen Cooling. Eastern Washington University Graduate Symposium (Cheney, Wa.) (oral presentation).